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DAMD17-00-1-0169**"Regulation of Apoptosis in Breast Cancer by NF- κ B and IAP Axis"****INTRODUCTION**

Apoptosis is a normal physiological cell suicide program that plays a fundamental role during development of multicellular organisms and in the maintenance of homeostasis in adults (1, 2). The control of cell number is achieved through a balance between cellular proliferation, differentiation, and apoptosis. Cancers arise when an imbalance occurs due to either inappropriate cellular proliferation or inhibition of apoptosis (3). Although prior attention has been given to the role of proliferation in cancer, an increasing body of evidence indicates that suppression of cell death underlies oncogenesis. This was first seen with bcl-2, an anti-apoptotic protein commonly over-expressed in cancers (4, 5). Upregulation of bcl-2 contributes to oncogenesis by inhibiting apoptosis, without apparent effects on cellular proliferation. Consistent with this concept, high levels of bcl-2 are seen in B cell lymphomas, prostate and colon cancers, and neuroblastoma. In contrast, in breast cancer cells, a reduced level of bcl-2 was observed compared to normal breast epithelium (6), suggesting the involvement of other apoptotic suppressors in the pathogenesis of breast cancer.

NF- κ B, apoptosis suppression, and multidrug resistance of tumor cells: Apoptosis plays a crucial role not only in the pathogenesis of cancers but also in tumor therapies associated with anti-cancer agents. Failure to undergo apoptosis has been implicated in resistance to chemotherapy in a variety of clinical models (7). Furthermore, the cytokine tumor necrosis factor- α (TNF- α) was identified as a cancer killer because of its ability to induce apoptosis in tumor cells. It was later found that most tumor cells are resistant to TNF- α -induced killing. Recently, the underlying molecular mechanism has been revealed. It is found that the transcription factor NF- κ B can protect cells from apoptosis (8-10). Paradoxically, TNF α , ionizing radiation, or chemotherapeutic compounds such as daunorubicin not only can trigger a cellular apoptosis pathway, but also activate NF- κ B.

NF- κ B is a family of dimeric transcription factors including RelA, c-rel, RelB, p50, and p52 subunits. In most cells, NF- κ B is sequestered in the cytoplasm as inactive forms by a family of inhibitory proteins termed I κ Bs that include I κ B α . Upon cellular stimulation with agents such as TNF- α , chemotherapeutic drugs, and ionizing radiation, I κ B proteins are rapidly degraded, thereby allowing NF- κ B translocation to the nucleus and activating target genes. In cells that express a dominant negative form of I κ B α , NF- κ B is irreversibly suppressed. Cells defective in NF- κ B activation exhibit enhanced apoptosis to stimuli such as TNF- α , daunorubicin, or ionizing radiation (8-10). We therefore undertook a search for novel genes that regulate NF κ B in breast cancers.

BODY

NF- κ B, apoptosis inhibition, and tumorigenesis

Recently, attention has been drawn to the transcription factor NF- κ B, a family of homo- or heterodimeric proteins characterized by the presence of the REL-homology domain (RHD), responsible for dimerization, DNA binding, nuclear localization and interaction with the ankyrin repeats of specific inhibitors of the I κ B family. Dysregulation of NF- κ B activity appears to contribute to a variety of pathologies, including inflammation and cancer. Moreover, evidence has been obtained suggesting that NF- κ B is at least partially responsible for chemoresistance, probably due to the anti-apoptotic function of NF- κ B, which transcriptionally up-regulates expression of several genes encoding apoptosis inhibitors, such as members of the Inhibitor of Apoptosis Proteins (IAP) family.

PAAD family proteins

The PAAD domain was recently identified in our lab as a novel protein module found at the N-termini of proteins involved in apoptosis, inflammation, cancer and immune responses (11). Other groups also identified the same protein domain and gave it different names (DAPIN, PYRIN) (12, 13).

The first 100 residues at the N-terminus of human, mouse and rat Pyrin protein are the prototype for this apparently vertebrate-specific domain. Pyrin was identified by genetic analysis as being responsible for Familial Mediterranean Fever (FMF), a hereditary hyper-inflammatory response syndrome (14). Recently, mutations in a new gene highly homologous to Pyrin (CIAS1), encoding the PAAD-containing protein Cryopyrin, have been shown to cause both familial cold autoinflammatory syndrome and Muckle-Wells syndrome (15, 16).

The PAAD domain is also present in a number of proteins involved in apoptosis and inflammation. These include: (a) CARD7/DEFCAP/NAC/NALP, a pro-apoptotic member of the Ced-4/Apaf-1 family; (b) Asc, a small proapoptotic protein that interacts with Pyrin; (c) the zebrafish homolog of Caspase 13; (d) a group of hematopoietic interferon-inducible genes that includes "myeloid nuclear differentiation antigen" (MNDA), "absent in melanoma 2" (AIM2) and "interferon- γ -inducible protein 16" (IFI16); and (e) several other as yet uncharacterized proteins predicted from the human genome draft sequence.

Based on computational secondary structure prediction and fold recognition, as well as circular dichroism, the PAAD domain is predicted to be organized in a 6 α -helix bundle and is considered the fourth member of the superfamily that includes death domains (DD), death effector domains (DED), and caspase recruitment domains (CARD).

KEY RESEARCH ACCOMPLISHMENTS

Our major accomplishments are summarized in two publications (17, 18). A brief summary follows:

- 1. Molecular cloning of human PAN2, a novel PAAD- and NACHT-containing protein.** Starting with the sequence of the 100-residue N-terminal region of the Pyrin protein, a cascade of

PSI-BLAST searches was performed, using new hits as queries for subsequent searches, until no new hits were found. The Saturated BLAST procedure allowed us to find several putative PAAD homologs in a number of uncharacterized proteins that we had predicted using the unfinished nucleotide database (HTGS, GSS, EST). These predicted proteins were confirmed and their sequence extended using the GENSCAN program for intron-exon prediction (19).

Interestingly, we found several proteins that, in addition to the PAAD domain, contain also the recently identified NACHT domain, which has been implicated in nucleotide binding and oligomerization (20). We termed these proteins PAN, for PAAD and NACHT. For one of these proteins, PAN2, we had only a partial cDNA prediction, encompassing the putative N-terminus of the gene but missing the C-terminus.

To verify the expression of PAN2 as a cDNA and to amplify it, a set of primers was designed on the basis of the predicted partial cDNA sequence, and used to perform RT-PCR using HeLa RNA. The PCR product was cloned into an expression vector (pcDNA3-Myc) and sequence-verified as containing nucleotides 1-1860 of PAN2.

To further define the expressed product of this gene, a BLAST search of the human EST database was performed using the partial cDNA sequence of PAN2 as a query. Several EST clones were identified that overlapped all or part of the PAN2 partial cDNA sequence. One of these clones contained an insert of 3.5 kbp and, after being completely sequenced, was found to contain the full-length Open Reading Frame (ORF) encoding the PAN2 protein, from the ATG start codon to the TAA stop codon (2985 bp), including the 3' UTR of the gene and the poly-A tail.

The protein encoded by PAN2 consists of 995 aminoacids and contains several distinct motifs, including a PAAD domain at the amino terminus (aa 1-89), a central NACHT domain (aa 147-465) and a C-terminal Leucine Rich Repeat (LRR) domain containing seven tandem LRRs.

2. Expression pattern of PAN2. To analyze the expression of PAN2 in different human tissues, first strand cDNAs from a variety of human tissues ([Clontech] panels I and II) were used as templates to amplify a region of PAN2 corresponding to the nucleotide-binding domain (aa 147-465). PAN2 is expressed at highest levels in spleen, but also in placenta, lung, liver, kidney, pancreas, breast, and thymus.

3. Regulation of NF- κ B activity by PAN2. As some members of the PAAD family proteins are involved in auto-inflammatory disorders, we asked whether PAN2 could regulate NF- κ B activation. To test this hypothesis, we transiently transfected HEK293 cells with a PAN2-encoding plasmid, together with a NF- κ B -dependent luciferase reporter gene plasmid. Cells were stimulated with IL-1 β or TNF- α , or co-transfected with signal transducers of the TNF- α pathway (TRAF2 and RIP), or of the IL-1 β pathway (MyD88, IRAK2 and TRAF6). Under these experimental conditions, PAN2 inhibited IL-1 β - and TNF α -induced NF- κ B activation, as well as NF- κ B induction by down-stream intracellular signal transducers involved in these cytokine signal transfection pathways (TRAF2, TRAF6, RIP, MyD88, IRAK). These observations imply that PAN2 interferes with a distal event at a point of convergence of these cytokine signal transduction pathways.

4. PAN2 suppresses NF- κ B DNA binding activity. To further explore the inhibitor effects of PAN2 on NF- κ B induction, a electro mobility shift assay (EMSA) was performed using ³²P-

labeled double-strand DNA oligonucleotides encompassing NF- κ B binding sites. Nuclear extracts were prepared from cells that had been transiently transfected with either control or *PAN2*-encoding plasmids, then stimulated with cytokines. Incubation of the nuclear extracts with 32 P-labeled NF- κ B probe then permitted measurements of the relative levels of the NF- κ B DNA binding activity.

TNF α and IL-1 β stimulated increases in NF- κ B DNA binding activity in control transfected cells. In contrast, NF- κ B DNA binding activity was markedly reduced in *PAN2* transfected cells. Incubating the protein/DNA complexes with various antibodies to members of the REL/ NF- κ B family of transcription factors provided evidence that P50 and P65 subunits of NF- κ B are included in the DNA/protein complexes detected by this EMSA. We conclude from these experiments that *PAN2* inhibits the induction NF- κ B DNA binding activity by cytokines.

5. *PAN2* inhibits the I κ B kinases (IKKs). Next, we examined the effects of *PAN2* overexpression on the I κ B kinase (IKK) complex. In this regard, the protein kinase directly responsible for the release of NF- κ B from its endogenous inhibitor I κ B is the IKK complex. This protein complex consists of three components, including IKK α , IKK β , and IKK γ . The IKK α and IKK β proteins are protein kinases that phosphorylate I κ B, thereby targeting it for poly-ubiquitination and subsequent degradation by the 26S proteasome. IKK γ is a scaffold protein that holds its complex together (21).

We therefore measured the relative activities and autophosphorylation of IKK α and IKK β in cells transiently transfected with *PAN2* encoding plasmids. For these experiments, HEK293 cells were transiently transfected with plasmids encoding either epitope-tagged IKK α or IKK β , together with either a control plasmid or a *PAN2*-encoding plasmid. After 24 hrs, cell lysates were prepared and IKK α or IKK β was immunoprecipitated. The resulting immunoprecipitates were then employed for in vitro kinase assays, where they were incubated with the endogenous substrate (GST-I κ B) in the presence of 32 P γ ATP. The kinase reaction products were then analyzed by SDS-PAGE, examining phosphorylation of GST-I κ B substrate as well as autophosphorylation of the IKK α and IKK β kinases. Furthermore, the immunoprecipitates were subjected to SDS-PAGE/immunoblot analysis to verify loading of equivalent amounts of proteins.

Cytokines induced increases in both the autophosphorylation and activity of IKK α and IKK β in control-transfected cells. In contrast, IKK α and IKK β activities were markedly reduced in *PAN2* over-expressing cells. These results indicate that *PAN2* interferes with the activation of the IKK complex. Studies are underway to attempt to elucidate the mechanism by which *PAN2* interferes with activation of this NF- κ B-inducing kinase complex.

REPORTABLE OUTCOMES

The reportable outcomes of this study include the discovery of a new gene, *PAN2*, and elucidation of its encoded proteins. In this regard, we have generated several critical new reagents that can also be viewed as reportable outcomes, including cDNAs encoding *PAN2* proteins engineered into expression vectors with epitope tags. We have also published a paper on *PAN2* (17) and another on related gene called NAC (18). In addition, Trainees supported by this grant participated in two additional publications on PAAD family proteins (11, 22).

CONCLUSIONS

Our goal to study mechanisms of NF- κ B regulations in breast cancers and in immune cells has lead us to the discovery of a new gene, PAN2, that appears to serve as an endogenous inhibitor NF- κ B induction. PAN2 is broadly expressed in normal human tissues, though found at somewhat higher levels in hematopoietic tissues. This multi-domain protein suppresses NF- κ B induction following stimulation of cells with several cytokines. Given that several intracellular mediators of cytokine-receptor signaling (that induce NF- κ B) are also inhibited by PAN2, it appears that this protein governs a common step at a point of convergence of several signal transduction pathways. Our data thus far point to the IKK complex as a likely site of action for the PAN2 protein. If so, PAN2 defines in novel type of regulator, which acts directly or indirectly on the IKK complex and interferes with its activity. We speculate, therefore, that over-expression of PAN2 in tumors could be used as a cancer-specific mechanism to prevent NF- κ B induction, and thereby reduce inflammatory and immune responses to tumors. Alternatively, reduced expression of PAN2 in tumors could be exploited as a mechanism for allowing increased activity of NF- κ B, which can have beneficial affects on tumors due to the ability of this transcription factor to induce the expression of several anti-apoptotic genes, including several members of the IAP family (22). Our current goals, therefore, are to elucidate the molecular mechanism of PAN2, and to define the patterns of PAN2 expression in normal and malignant mammary tissues, correlating that with the expression of NF- κ B inducible members of the IAP family. All together, these results should provide new insights into mechanisms of tumor/immune system interaction and apoptosis suppression.

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ortholog of the human ASC protein; the other is an ICE (interleukin-1 β -converting enzyme)-like protease. The combination of the DAPIN with ICE-like protease p10 and p20 domains is consistent with the assumption of an adapter function for DAPIN that complements the protease effector function in this novel caspase.

The identification of a common DAPIN domain links a well-characterized family of nuclear interferon-inducible proteins to other proteins with putative functions in apoptosis and tumor biology, viral infection and inflammation. Future research on DAPIN proteins should reveal the physiological and biochemical function of this domain and the small viral DAPIN proteins might be especially helpful for this task.

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PAAD – a new protein domain associated with apoptosis, cancer and autoimmune diseases

Krzysztof Pawłowski, Frederic Pio, Zhi-Liang Chu, John C. Reed and Adam Godzik

A new protein domain was found in several proteins involved in apoptosis, inflammation, cancer and immune responses. Its location within these proteins and predicted fold suggests that it functions as a protein–protein interaction domain, possibly uniting different signaling pathways.

The general framework of apoptosis was elucidated by studies of the nematode *Caenorhabditis elegans*, where apoptosis regulation is achieved by the interplay of only four proteins, namely EGL-1, CED-3, CED-4, and CED-9. In humans, the catalog of known apoptosis-relevant proteins lists hundreds of proteins and is rapidly expanding. However, analysis of these

proteins is greatly aided by dividing the larger proteins into discrete domains with known functions and structural features. Many domains involved in apoptosis are identifiable by sequence comparisons in simple lower organisms, where their functions can be more easily studied¹.

Here, we report the discovery of a new domain, PAAD, named after the protein families from which it was discovered: pyrin, AIM (absent-in-melanoma), ASC [apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)], and death-domain (DD)-like. This 80–100-residue domain is present at the N terminus of several different proteins involved in apoptosis, inflammation,

cancer and immune responses. Secondary structure predictions have identified the PAAD domain as mostly α -helical (Fig. 1).

Starting with the sequence of the pyrin protein (see below), a cascade of PSI-BLAST (Ref. 2) searches was performed, using new hits as queries for subsequent searches, until no new hits were found (see Ref. 3). Lower-significance hits from this procedure (called Saturated BLAST) were confirmed using the profile-to-profile alignment algorithm FFAS (Ref. 4) against a library of apoptosis-related domains. Proteins suspected of having a PAAD domain were added to the Saturated BLAST and FFAS databases, and the FFAS similarity score

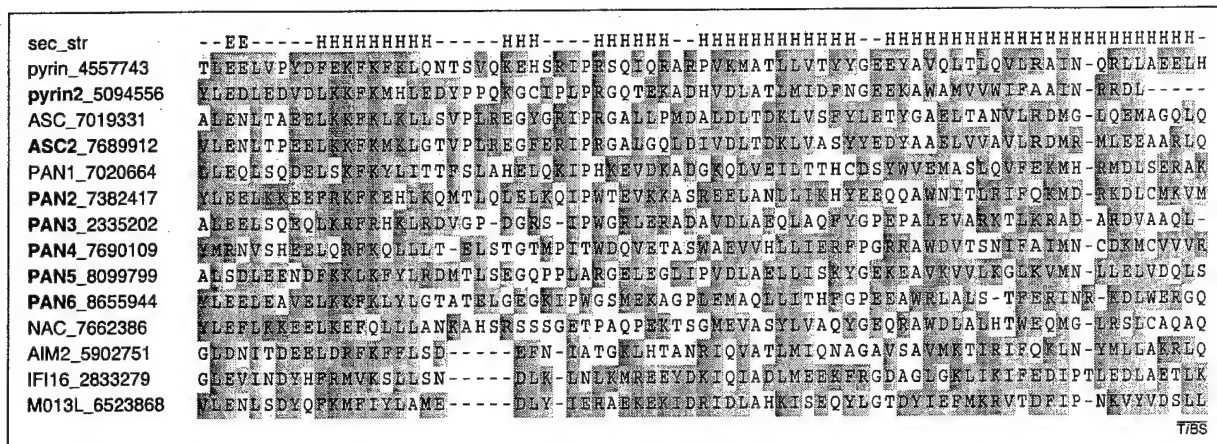


Fig. 1. Multiple alignment (from the CLUSTAL W program¹⁹) of the aligned part of selected members of the PAAD family from humans. NCBI gi accession numbers are included. PAAD domains from putative proteins predicted from nucleotide databases are shown in bold. The 'sec_str' line shows secondary structure prediction made for pyrin using the PHD program²⁰. Abbreviations: E, β strand; gi, GenBank identifier; H, α helix; NCBI, National Center for Biotechnology Information; PAAD, from pyrin, AIM (absent-in-melanoma), ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) and death-domain (DD)-like.

was used to accept or reject the putative PAAD domains. Most of the proteins identified in Fig. 1 were associated with each other with a PSI-BLAST expectation value, E , of <0.001 or a FFAS Z -score of >10 , or both. The weakest association was between the AIM2/IFI16 (interferon-inducible protein 16) branch and the rest of the family (pyrin/ASC/caspase/NAC), with $E=0.05$ and FFAS Z -scores between 6 and 10. The latter values correspond to a significant similarity. In a test on a large group of proteins with known three-dimensional structures and dissimilar structures (a structure benchmark), more than 99% of pairs with pairwise sequence scores of >8 had similar three-dimensional structures⁴. The same link was also confirmed by independent application of the Gibbs sampling algorithm⁵, where sequence patterns identified in the pyrin/ASC/caspase branch of the family could be used consistently to find the AIM2/IFI16 group, albeit with low significance.

The first 100 residues of the N terminus of the pyrin protein were used to define the prototype of the PAAD family. Pyrin, a large, 645-residue protein was identified by genetic analysis as being responsible for familial Mediterranean fever (FMF), a hereditary hyper-inflammatory response syndrome⁶. Several mutations that cause FMF were identified in the C-terminal half of pyrin. The N-terminal region, which contains the PAAD domain, was not characterized. Pyrin homologs were also identified in mouse, rat, cow and zebrafish.

The PAAD domain was also found to be present in the N-terminal region of AIM2

and several closely homologous human and murine proteins, such as those encoded by interferon-inducible genes *IFI16* and *MNDA* (Ref. 7). Proteins from this family contain one or more copies of a conserved 200-residue IFI20X domain, which is implicated in transcription repression⁸. With two exceptions, the N-terminal region of AIM2 and related proteins that contain the PAAD domain has not been functionally analyzed. In MNDA protein, the N-terminal domain was shown to be partly responsible for homodimerization⁹ and in IFI16, DNA-binding was attributed to a 159-residue N-terminal region¹⁰. Two viral proteins homologous to the proteins from the interferon-inducible MNDA or IFI16 family were also identified: M013L from myxoma virus and gp013L from rabbit fibroma virus. These contain only the PAAD domain.

The PAAD domain is also present at the N-terminus of the recently identified gene from zebrafish, which is homologous to the apoptotic protease, caspase¹¹. In this protein, the PAAD domain occupies a position corresponding to the prodomain, which in other initiator caspases is occupied by a CARD or a death effector domain (DED). This supports the possible involvement of the PAAD domain in apoptosis.

Finally, the PAAD domain was also found in the N terminus of the ASC protein¹². This protein was identified by characteristic dot-like aggregates (specks) present in cells during apoptosis triggered by retinoic acid and other anti-cancer drugs¹². The C-terminal half of the ASC protein contains an easily recognizable CARD domain, whereas the N-terminal

half, occupied by a PAAD domain, has been referred to as a pyrin-like domain¹². Homologs of the ASC protein were identified in zebrafish, pufferfish and cow.

The Saturated BLAST procedure also allowed us to find several putative PAAD homologs in a number of uncharacterized proteins that we had either predicted using the unfinished nucleotide databases (HTGS, GSS, EST) or, in some cases, identified in the non-redundant (NR) database. These predicted proteins were confirmed and their sequences extended using the GENSCAN program¹³ for exon prediction. A near-complete list of human proteins with identified PAAD domains is presented in Fig. 1. Interestingly, the list includes seven proteins that contain domains similar to the recently identified NACHT (named after NAIP, CIA, HET-E and TP1 proteins) nucleotide-binding family¹⁴, in addition to the PAAD domain (Fig. 2). The NACHT domain has been implicated in nucleotide binding and protein oligomerization. We termed these proteins PAN, for PAAD and nucleotide-binding. Other putative PAAD domain proteins include human ASC2, an 89-residue protein that consists solely of the PAAD domain.

One of the putative proteins from the unfinished genomic databases, namely PAN6, allowed an independent and unambiguous connection between the pyrin/ASC/caspase and the AIM2/IFI16 branches of the family. Three iterations of a standard PSI-BLAST search against the National Center for Biotechnology Information (NCBI) NR database seeded with this putative domain reveal

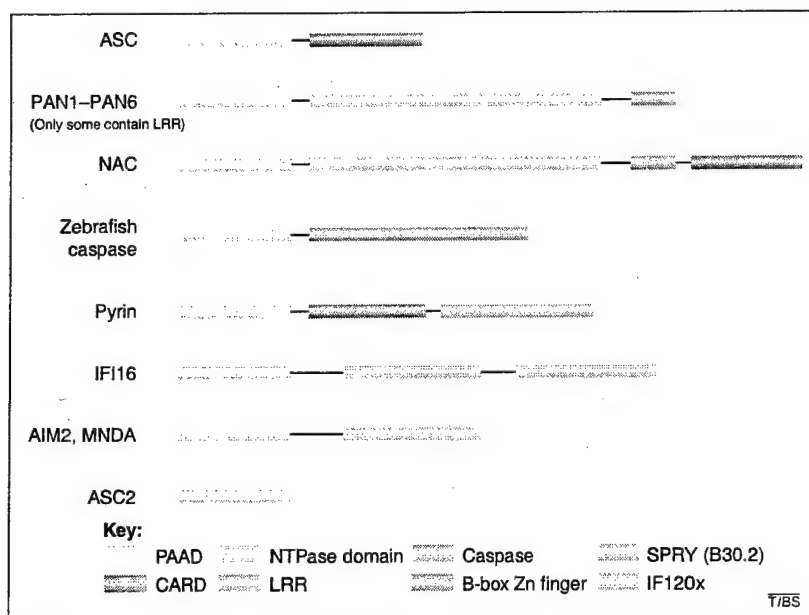


Fig. 2. Domain arrangement in proteins that possess a PAAD domain (not to scale). Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD); IFI16 or IFI20x, interferon-inducible protein 16 or 20x, respectively; LRR, leucine-rich repeats; MNDA, myeloid cell nuclear differentiation antigen; NAC, nucleotide binding and CARD (caspase recruitment domain); PAAD, from pyrin, AIM (absent in melanoma), ASC and DD (death-domain)-like; PAN1, PAAD and nucleotide-binding protein 1; SPRY, SP1a and Ryanodine receptor.

homology with pyrin and AIM2. The average sequence similarity between different branches of the family is quite low, but clear regions of strong similarity are conserved throughout the family, most notably the KFKxxL pattern, which is present in the N-terminal half of the PAAD domain (Fig. 1).

Without more direct characterization of the PAAD domain it is difficult to speculate about its possible role. However, its presence in proteins involved in apoptosis and immune disorders raises the possibility that this domain can somehow couple these two processes. This idea can be taken further by considering the results of several publicly available fold-recognition algorithms, which independently predict that the PAAD domain has a fold compatible with the fold of DD/DED/CARD, consisting of a six- α -helical bundle. These domains are responsible for specific interactions among proteins participating in apoptosis regulation¹.

Five independent methods using different types of information and criteria were used to identify the nature of the PAAD fold: FFAS (Ref. 4) (sequence-only, profile-profile alignment), GenThreader¹⁵ (classical threading based on statistical potentials combined by means of neural networks with heuristic criteria), SAM-99 (Ref. 16) (sequence-only, Hidden Markov Models),

bioinbu¹⁷ (sequence and sequence-derived features) and 3D-PSSM (Ref. 18) (sequence features plus statistical potentials). All these methods identified the DD fold as the one most like that of the PAAD domain, albeit with relatively low significance. For example, FFAS predicted the DD fold with a Z-score of 6.09 (Protein Data Bank code 1fadA).

Fold-prediction results could imply that the PAAD domain forms a fourth branch of the DD/DED/CARD-fold family, with a similar structural framework being used to facilitate recognition among groups of proteins that contain this domain. Fold prediction and modeling studies for the PAAD domain will be the subject of a separate publication.

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A Novel PAAD-containing Protein That Modulates NF- κ B Induction by Cytokines Tumor Necrosis Factor- α and Interleukin-1 β *

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PAAD domains are found in diverse proteins of unknown function and are structurally related to a superfamily of protein interaction modules that includes death domains, death effector domains, and Caspase activation and recruitment domains. Using bioinformatics strategies, cDNAs were identified that encode a novel protein of 110 kDa containing a PAAD domain followed by a putative nucleotide-binding (NACHT) domain and several leucine-rich repeat domains. This protein thus resembles Cryopyrin, a protein implicated in hereditary hyperinflammation syndromes, and was termed PAN2 for PAAD and NACHT-containing protein 2. When expressed in HEK293 cells, PAN2 suppressed NF- κ B induction by the cytokines tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β), suggesting that this protein operates at a point of convergence in these two cytokine signaling pathways. This PAN2-mediated suppression of NF- κ B was evident both in reporter gene assays that measured NF- κ B transcriptional activity and electromobility shift assays that measured NF- κ B DNA binding activity. PAN2 also suppressed NF- κ B induction resulting from overexpression of several adapter proteins and protein kinases involved in the TNF or IL-1 receptor signal transduction, including TRAF2, TRAF6, RIP, IRAK2, and NF- κ B-inducing kinase as well as the I κ B kinases IKK α and IKK β . PAN2 also inhibited the cytokine-mediated activation of IKK α and IKK β as measured by *in vitro* kinase assays. Furthermore, PAN2 association with IKK α was demonstrated by co-immunoprecipitation assays, suggesting a direct effect on the IKK complex. These observations suggest a role for PAN2 in modulating NF- κ B activity in cells, thus providing the insights into the potential functions of PAAD family proteins and their roles in controlling inflammatory responses.

The PAAD¹ domain is found in diverse proteins implicated in apoptosis, inflammation, and cancer (1). This protein fold,

which is also known as PYRIN or DAPIN (2–4), is predicted to form an α -helical bundle resembling death domains, death effector domains, and Caspase activation and recruitment domains (CARDs). The PAAD domain thus constitutes the fourth branch of this superfamily of structurally similar protein modules, which participate in homotypic protein-protein interactions involved in signal transduction by tumor necrosis factor (TNF) family cytokine receptors and pathways connected to activation of Caspase family cell death proteases and to kinases important for induction of NF- κ B family transcription factors (for reviewed, see Ref. 5).

The founding member of this family of proteins is Pyrin, which contains a PAAD domain at its N terminus followed by a B-box zinc finger and SPRY domain, a motif found in ryanodine receptors that is involved in Ca²⁺ release (6). Pyrin was identified by genetic analysis of families affected with familial Mediterranean fever, a hereditary hyperinflammatory response syndrome (7). The mutations identified in familial Mediterranean fever patients fall within the region C-terminal to the PAAD domain. Recently hereditary mutations have been identified in the CIAS gene, which encodes the PAAD-containing protein Cryopyrin (8). These mutations are predicted to produce mutant Cryopyrin proteins in patients affected with familial cold autoinflammatory syndrome and Muckle-Wells syndrome with mutations residing downstream of the N-terminal PAAD domain. Thus, PAAD-containing proteins appear to regulate pathways relevant to inflammation, although the molecular mechanisms are unknown. Providing further evidence of a potential link to inflammatory cell function, PAADs are also found in a group of interferon-inducible genes that includes myeloid nuclear differentiation antigen, absent in melanoma 2, and interferon- γ -inducible protein 16.

PAAD-containing proteins have also been implicated in apoptosis regulation. The PAAD domain, for example, is present in (a) ASC, a proapoptotic adapter protein that contains both a PAAD and a CARD and that interacts with Pyrin (9), and (b) NAC (CARD7/DEFCAP/NALP), an apoptosis-promoting protein that contains PAAD and CARD modules (together with additional domains) and that reportedly enhances activation of Caspase-9 either directly or indirectly through interactions with the Caspase-9 activator Apaf1 (4, 10, 11). A zebrafish Caspase also contains a PAAD within its N-terminal prodomain (12), suggesting possible links of PAADs to apoptosis. However, no evidence of direct involvement of PAAD domains in apoptosis has been obtained to date.

In total, at least 34 genes encoding PAAD-containing pro-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY072792.

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¹ The abbreviations used are: PAAD, Pyrin/absent in melanoma/ASC/death domain-like; NACHT, NAIP, CIAP, HET-E, TP-1; CARD, caspase activation and recruitment domain; TNF, tumor necrosis factor; IL,

interleukin; EMSA, electromobility shift assay; IKK, I κ B kinase; LRR, leucine-rich repeat; EST, expressed sequence tag; HTGS, High Throughput Genome Sequences; aa, amino acid(s); HA, hemagglutinin; GST, glutathione S-transferase; Nik, NF- κ B-inducing kinase.

teins are predicted to reside in the human genome (1).² Fourteen of these PAAD family proteins, including Cryopyrin, have a conserved architecture that includes an N-terminal PAAD followed by a nucleotide-binding fold known as the NACHT domain (13) and then variable numbers of leucine-rich repeat (LRR) domains as well as other domains in some members. We have termed these proteins PANs for PAAD and NACHT domain proteins. The topological organization of domains in the PANs is reminiscent of proteins previously implicated in NF- κ B induction or Caspase activation such as Nod1 (CARD4), Nod2 (inflammatory bowel disease protein 1), and CLAN (Ipafl, CARD12), which contain a CARD followed by NACHT and LRR domains (14–16). In those proteins, the N-terminal CARD is essential for the effector functions of these proteins as inducers of NF- κ B or activators of Caspases. In this report, we explored the effects of one of the members of this family, PAN2, finding evidence that this protein modulates NF- κ B induction via its PAAD domain.

MATERIALS AND METHODS

Bioinformatics—Using the sequence of the 100-residue N-terminal region of the PIRIN protein, a cascade of PSI-BLAST searches was performed using new hits as queries for subsequent searches until no new hits were found. This procedure, called Saturated BLAST (17), revealed several genomic loci and EST clones potentially capable of encoding PAAD domains in the publicly available nucleotide databases (HTGS, Genome Survey Sequences, EST, and draft human genome). For genomic data, the amino acid sequences of the predicted PAAD-containing proteins were tentatively deduced using the GENSCAN program for intron-exon prediction (18). Several examples were found of proteins that are predicted to contain a PAAD domain together with a NACHT domain, thus constituting members of the PAN family. In this report, we describe PAN2, which is encoded within the genomic locus AC022066 on chromosome 19 with partial or complete open reading frames for this protein encompassed in representative EST clones BE018433, AA421452, and AI204456.

Plasmids—Plasmids were generated using PCR procedures with primers designed to incorporate appropriate restriction enzyme sites. Polymerase chain reaction products were then digested and cloned into pcDNA3Myc vector. All plasmids were sequence-verified.

Reverse Transcriptase-PCR Assays—Panels of first-strand cDNAs (CLONTECH Panel I and II) generated from the mRNA of various human tissues were used as templates to amplify a region of PAN2 corresponding to the nucleotide-binding domain (amino acids (aa) 147–465) using the forward and reverse oligonucleotides 5'-CCACGTACAGTGATTATTCAGGACC-3' and 5'-CAATAGAACAGGCGGCACAG-3', respectively. Alternatively, total RNA from HeLa cells was subjected to reverse transcriptase-PCRs to amplify a partial clone of PAN2 (aa 1–620) using the following oligonucleotides: forward, 5'-ATGGCAGCCTCTTCTCTCTGATTCT-3'; reverse, 5'-CGACGTAGAGCTGTGTTTCATCTCTTCTTAA-3'. The resulting PCR product was excised from agarose gels and cloned into pcDNA3Myc, and its identity was confirmed by DNA sequencing.

Antibody Generation and Immunoblot Analysis—A polyclonal anti-PAN2 antiserum was generated by repeated immunization of rabbits with an 18-mer synthetic peptide spanning aa 139–157 of PAN2 protein (FAPKETGKQPRVTVIIQGPQ) conjugated to maleimide-activated carrier proteins keyhole limpet hemocyanin and ovalbumin (Pierce). Total protein lysates from various untransfected cell lines, from 293 cells transiently transfected with Myc-PAN2 (as a positive control), and from stably transfected 293-Neo and 293-PAN2 cell lines were prepared, normalized for total protein content (100 μ g), and size-fractionated in an 8% polyacrylamide gel under standard SDS-PAGE conditions. Proteins were then transferred onto nitrocellulose membranes (Bio-Rad) and incubated with a 1:1000 (v/v) dilution of anti-PAN2 antiserum or anti- β -actin antibody (Sigma). Detection was accomplished by enhanced chemiluminescence.

Luciferase Reporter Gene Assays—Typically 1×10^4 HEK293 cells were plated in 96-well plates and transfected using Superfect transfection reagent (Qiagen) following the manufacturer's recommended protocol. For NF- κ B reporter assays, cells were transfected with 50 ng of pNF- κ B-luc and 10 ng of pTK-RL reporter vectors (Stratagene) and

various amounts of the relevant expression plasmids as described in the figure legends, maintaining the total amount of DNA constant using pcDNA3Myc empty vector. The β -catenin reporter assays were performed in the same way using 100 ng of β -catenin reporter plasmid, 10 ng of pTK-RL, and 500 ng of β -catenin expression vector (19). At 36 h after transfection, cells were treated with TNF α or IL-1 β (both 20 ng/ml) for 6 h where indicated. Activities from firefly and *Renilla* luciferases were assayed using the Dual-Luciferase Reporter Assay System (Promega).

Stable Transfections—For stable transfections, 5×10^5 HEK293 cells were seeded in 6-cm plates and transfected with 2.5 μ g of pMyc-PAN2 or pMyc empty vector using LipofectAMINE Plus (Invitrogen). After 2 days, transfected cells were split 1:3 into 10-cm dishes and grown in the presence of 1 mg/ml G418 until individual colonies appeared. Several well separated clones were recovered and analyzed by immunoblotting to assess relative levels of PAN2. One clone expressing PAN2 was identified (293-PAN2) and used to perform electromobility shift assays (EMSAs). A clone transfected with pcDNA3Myc plasmid (293-Neo) was also randomly picked for use as a control.

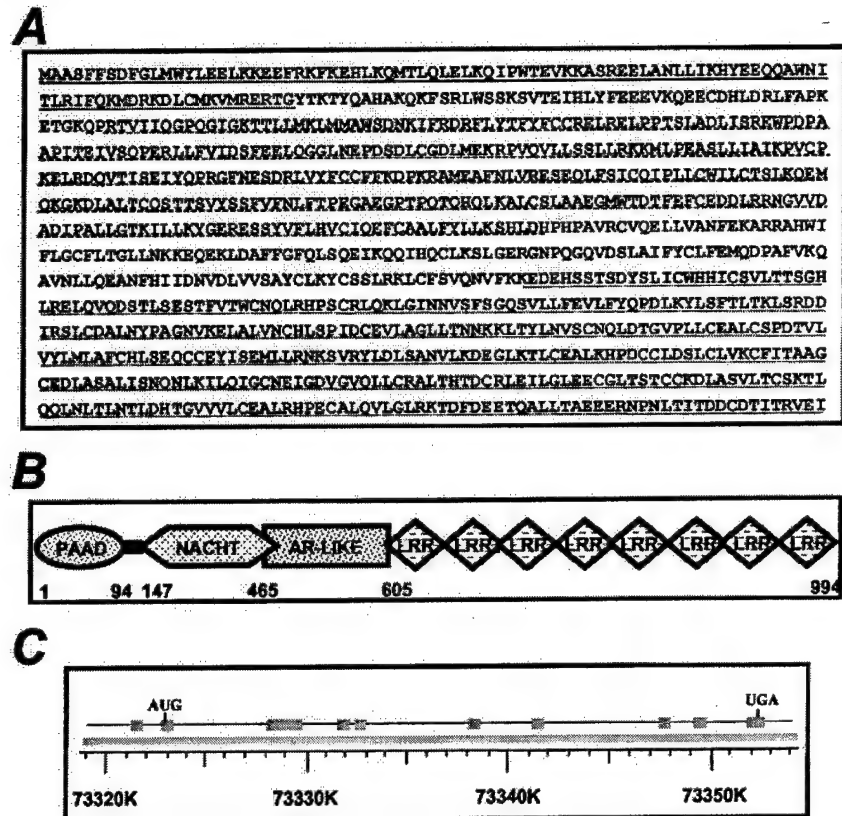
Co-immunoprecipitation Assays—293T cells were seeded at a density of 2×10^6 cells/10-cm plate the day before transfection. Cells were transfected with 6 μ g of various combinations of plasmids as described above and then harvested 36 h after transfection. Alternatively stably transfected 293-Neo and 293-PAN2 cells were used. Cells were lysed in 400 μ l of immunoprecipitation lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol) containing protease inhibitors (Roche Molecular Biochemicals). After centrifugation, clarified lysates were subjected to immunoprecipitation with anti-HA or anti-Myc antibodies immobilized on agarose (Santa Cruz Biotechnology) for 3 h at 4 °C followed by three washes in immunoprecipitation lysis buffer. Immune complexes were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and analyzed by immunoblotting using anti-Myc or anti-I κ B kinase α (IKK α) antibodies (Santa Cruz Biotechnology).

EMSAs—Stably transfected 293-Neo and 293-PAN2 cells were seeded in 10-cm plates, cultured overnight in 1% fetal bovine serum, and then left untreated or treated with 20 ng/ml TNF α for 30 min or with 100 ng/ml IL-1 β for 2 h. Nuclear extracts were prepared from these cells, and EMSAs were carried out as described previously (20). Briefly, a double-stranded oligonucleotide containing a consensus NF- κ B binding site (Promega) was end-labeled with [γ -³²P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Amersham Biosciences). After purification with MicroSpin G-25 columns (Amersham Biosciences), the labeled probe (15 fmol) was incubated with 2 μ g of nuclear extracts for 25 min at room temperature. The nuclear extracts from TNF-treated cells were also incubated with specific antibodies recognizing the NF- κ B subunits p65 and p50 (Santa Cruz Biotechnology) before the binding reaction or with 100-fold molar excess of unlabeled DNA probe as specific competitor. All complexes were separated by electrophoresis in non-denaturing 5% polyacrylamide gels at 4 °C. After drying, gels were exposed to x-ray film at -70 °C.

Kinase Assays—293 cells were transfected with 0.5 μ g of FLAG-IKK α expression vector and either pMyc empty vector, increasing amounts of pMyc-PAN2, or 0.5 μ g of the indicated Myc-tagged PAN2 deletion mutants. At 36 h after transfection, cells were left untreated or treated with 20 ng/ml TNF α for 15 min. Cells were resuspended in kinase assay lysis buffer (50 mM Tris (pH 7.5), 200 mM NaCl, 2 mM EDTA, 1% Brij 97 (polyoxyethylene alcohol), 10% glycerol, 0.5% Triton X-100) supplemented with a mixture of protease inhibitors (Roche Molecular Biochemicals) and 2 mM phenylmethanesulfonyl fluoride- α -toluenesulfonyl fluoride, 50 μ M dithiothreitol, and 1 mM Na₃VO₄. Lysates were immunoprecipitated with anti-FLAG antibody conjugated to agarose (Sigma) for 3 h at 4 °C. The immunoprecipitates were washed three times in lysis buffer and then washed once with kinase buffer (20 mM Hepes (pH 7.4), 5 mM MgCl₂, 2 mM MnCl₂, 1 mM dithiothreitol, 0.1 mM Na₃VO₄, 20 mM NaF, and 10 mM β -glycerophosphate). Kinase assays were performed in 15 μ l of kinase buffer containing 5 μ M ATP, 5 μ Ci of [γ -³²P]ATP and 1 μ g of GST-I κ B α -(1–54) for 20 min at 30 °C. Reactions were stopped by adding 3 μ l of 5 \times Laemmli buffer and boiling for 5 min. Samples were subjected to 12% SDS-PAGE, transferred to nitrocellulose membranes, and exposed to x-ray film. The same membranes were subsequently probed with anti-FLAG antibody to determine the amounts of FLAG-IKK α present. Cell lysates were normalized for total protein content (30 μ g) and analyzed by immunoblotting to confirm expression of Myc-PAN2 and Myc-tagged PAN2 mutants.

² A. Godzik and J. C. Reed, unpublished data.

FIG. 1. Predicted amino acid sequence and genomic structure of PAN2 gene. **A**, the predicted amino acid sequence of the 994-aa PAN2 protein is presented. The PAAD, NACHT, and LRRs are indicated by underlining using solid, dashed, and dotted lines, respectively. **B**, schematic diagram of the structural features of PAN2. The PAAD domain comprises aa 1–94, the NACHT domain aa 147–465, and the LRR domain aa 605–995. Between the NACHT and LRRs is a domain that is commonly associated with NACHT-containing proteins and shares sequence similarity with a putative angiotensin receptor (AR) (L. Jaroszewski and A. Godzik, unpublished). **C**, the exon-intron organization of the PAN2 gene is presented as deduced from cDNA sequencing and comparison to the Human Genome Database genomic sequence.



RESULTS

Molecular Cloning of Human PAN2, a Novel PAAD- and NACHT-containing Protein—Using bioinformatics strategies, we identified several genes potentially capable of encoding predicted proteins having PAAD and NACHT domains, thus constituting members of the PAN family, which includes NAC, Cryopyrin, PAN2, and 11 other related proteins, all sharing significant sequence homology. An HTGS clone (accession number AC022066) allowed us to obtain genomic sequence data for PAN2, and the analysis of this HTGS clone with the GENSCAN program provided an intron-exon prediction encompassing the N-terminal segment of PAN2. To independently verify expression of PAN2, a set of primers was designed based on the predicted partial cDNA sequence and used to perform reverse transcriptase-PCR using HeLa cell RNA. The PCR product was cloned into a plasmid (pcDNA3Myc) and sequenced, verifying that it contained nucleotides 1–1860 of the PAN2 cDNA.

To further define the expressed product of the PAN2 gene, a BLAST search of the human EST database was performed using this partial cDNA as a query. Several EST clones were identified that overlapped all or part of the PAN2 partial cDNA sequence. One of these clones contained an insert of 3.4 kbp and was obtained for complete sequencing, revealing an open reading frame of 2985 nucleotides encoding the 994-aa PAN2 protein (Fig. 1A). The authenticity of this open reading frame was confirmed by *in vitro* translation (not shown). The initiating AUG codon of this open reading frame was preceded by a 5'-untranslated region of 57 bp, whereas the stop codon (2985 bp) was followed by a 3'-untranslated region of 348 bp and a poly(A) tail (submitted to GenBankTM, accession number AY072792). This cDNA is substantially similar to a recently deposited sequence (GenBankTM accession number AF442488). The predicted PAN2 protein contains a PAAD domain at its N

terminus (aa 1–94), a central NACHT domain (aa 147–465), and eight tandem C-terminal LRR domains (aa 605–995) (Fig. 1B).

Using the cDNA sequence of the 3.4-kbp PAN2 clone, we deduced the exon-intron organization of the gene (Fig. 1C). The PAN2 gene spans 30 kbp on chromosome 19 and contains at least 10 exons, including 9 coding and at least 1 non-coding exon (the possibility of additional 5'-untranslated region exons cannot be excluded). Interestingly, none of the standard gene prediction programs was able to predict the complete PAN2 cDNA sequence from genomic data. Most often the PAAD-containing exon is predicted to be part of an intron or a promoter region in an upstream gene coding for an uncharacterized protein from the Mucin family. Since the Mucin family contains multiple copies of a 50-aa repeat, it is possible that the genomic sequence in this region was not assembled correctly.

To analyze the expression of PAN2, first-strand cDNAs generated from equivalent amounts of RNA from a variety of normal human tissues were used as templates to amplify a region of the PAN2 cDNA corresponding to the NACHT domain (aa 147–465). This analysis revealed that PAN2 is expressed at highest levels in spleen but also in placenta, lung, liver, kidney, pancreas, and thymus (Fig. 2A).

We also generated a polyclonal antiserum raised against a peptide corresponding to aa 139–157 of PAN2. This antiserum recognized PAN2 as a single band of ~110 kDa (which is in agreement with the predicted molecular mass of 113.4 kDa). The slower migration of plasmid-derived PAN2 compared with the endogenous protein is due to the Myc epitope tag appended to the latter (Fig. 2B). Immunoblot analysis of a panel of human cell lines of various tissue origins revealed widespread expression of the PAN2 protein (Fig. 2C).

Regulation of NF- κ B Activity by PAN2—We sought evidence that PAN2 might modulate activation of Caspases involved in either apoptosis or inflammation but failed to observe consist-

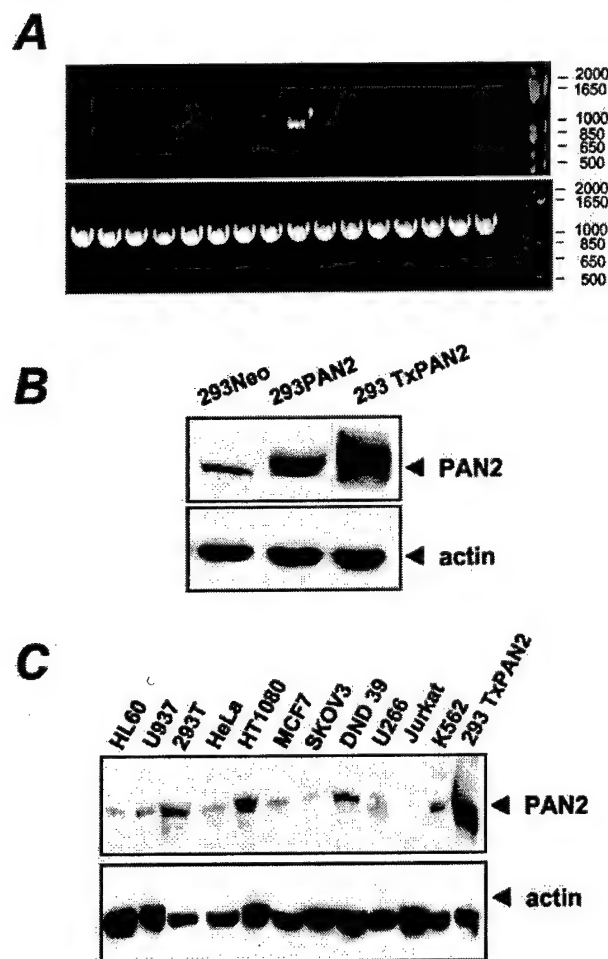


Fig. 2. PAN2 expression in human tissues and cell lines. A, first-strand cDNAs (CLONTECH) were used as templates for amplifying either a region of PAN2 corresponding to the NACHT domain (aa 147–465) (top) or glyceraldehyde-3-phosphate dehydrogenase as a control (bottom). PCR products were analyzed by agarose gel electrophoresis and visualized by UV illumination of ethidium bromide-stained gels. Lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas; lane 9, spleen; lane 10, thymus; lane 11, prostate; lane 12, testis; lane 13, ovary; lane 14, intestine; lane 15, colon; lane 16, peripheral blood lymphocytes; lane 17, negative control reaction performed without cDNA. Molecular weight markers are shown at right in bp. B, total protein lysates (80 μ g) were prepared from stably transfected 293-Neo and 293-PAN2 (lanes 1 and 2) and from 293 cells transiently transfected with Myc-PAN2 plasmid (lane 3) and analyzed by immunoblot for PAN2 protein expression. The same membrane was then stripped and reprobed for β -actin as a loading control. C, total protein lysates (100 μ g) were prepared from the indicated human cell lines and from 293 cells transiently transfected with Myc-PAN2 plasmid (positive control, rightmost lane) and analyzed as in B.

ent effects in transient transfection assays (not shown). Because some members of the PAAD family proteins are involved in hyperinflammatory diseases (21), we asked whether PAN2 could regulate NF- κ B induction given that activation of this family of heterodimeric transcription factors is commonly involved in immune and inflammatory cell biology (for review, see Ref. 22). To explore this possibility, we transiently transfected HEK293 cells with a PAN2-encoding plasmid together with a NF- κ B-dependent luciferase reporter gene plasmid. Cells were stimulated with IL-1 β or TNF α , or they were co-transfected with plasmids encoding various signal transducing proteins within the TNF α receptor (TRAF2 and RIP) or the IL-1 β receptor/Toll family receptor (MyD88, IRAK2, and

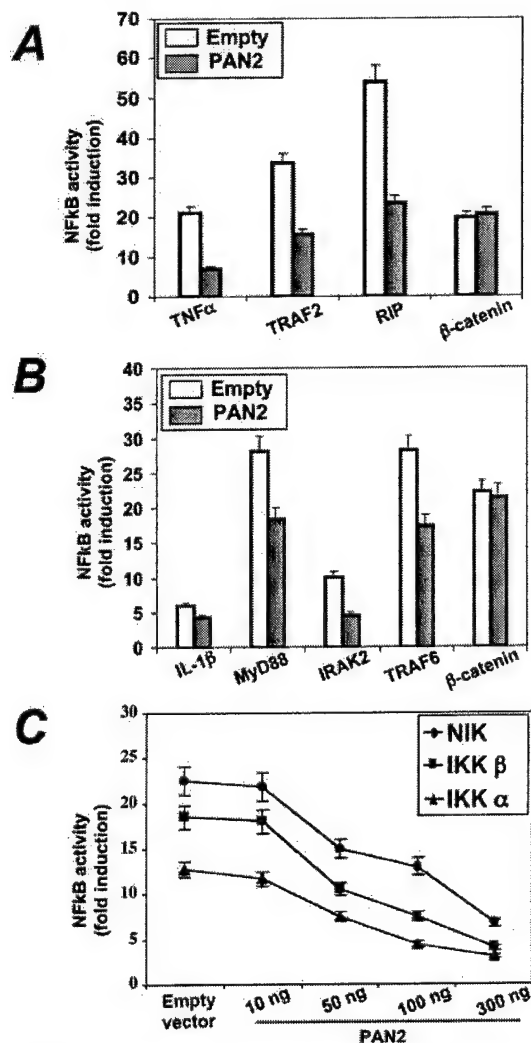


Fig. 3. PAN2 inhibits NF- κ B induction. A and B, HEK293 cells were seeded into 96-well plates and transfected on the following day with 50 ng of pNF- κ B-luc and 10 ng of pTK-RL reporter gene plasmids together with 400 ng of pcDNA3Myc empty vector (Empty, white bars) or 400 ng of pcDNA3Myc-PAN2 (PAN2, gray bars) and stimulated for 6 h with TNF α (A) or IL-1 β (B). Alternatively cells were co-transfected with 100 ng of plasmids encoding TRAF2, TRAF6, MyD88, RIP, or IRAK2. The last bars in each panel represent a control in which 100 ng of β -catenin-luc reporter gene plasmid and 500 ng of β -catenin-encoding plasmid were transfected. After 36 h, cells were harvested, and the ratio of firefly to *Renilla* luciferase activity was determined for each sample. Numbers indicate fold induction of the NF- κ B reporter gene above base line (mean \pm S.D., $n \geq 3$). C, HEK293 cells were co-transfected with 50 ng of pNF- κ B-luc and 10 ng of pTK-RL reporter gene plasmids together with 80 ng of pcDNA3Myc-Nik, pcDNA3HA-IKK α , or pcDNA3HA-IKK β and with either pcDNA3Myc empty vector or various amounts of pcDNA3Myc-PAN2 (ranging from 10 to 300 ng), holding total DNA constant at 360 ng per transfection. Luciferase assays were performed 36 h after transfection as described above (mean \pm S.D., $n \geq 3$).

TRAF6) pathway (for review, see Ref. 23). Under these experimental conditions, PAN2 potently inhibited NF- κ B induction by TNF α (Fig. 3A) and to a lesser extent by IL-1 β (Fig. 3B). PAN2 overexpression also markedly reduced NF- κ B activity induced by intracellular adapter proteins (TRAF2, TRAF6, and MyD88) and kinases (RIP and IRAK2) that functionally connect TNF and IL-1R receptors to NF- κ B responses (Fig. 3, A and B). These effects of PAN2 were specific in that the activity of other transcription factors such as β -catenin and p53 were not suppressed (Fig. 3 and data not shown). Moreover, immunoblotting confirmed that PAN2 did not interfere with the

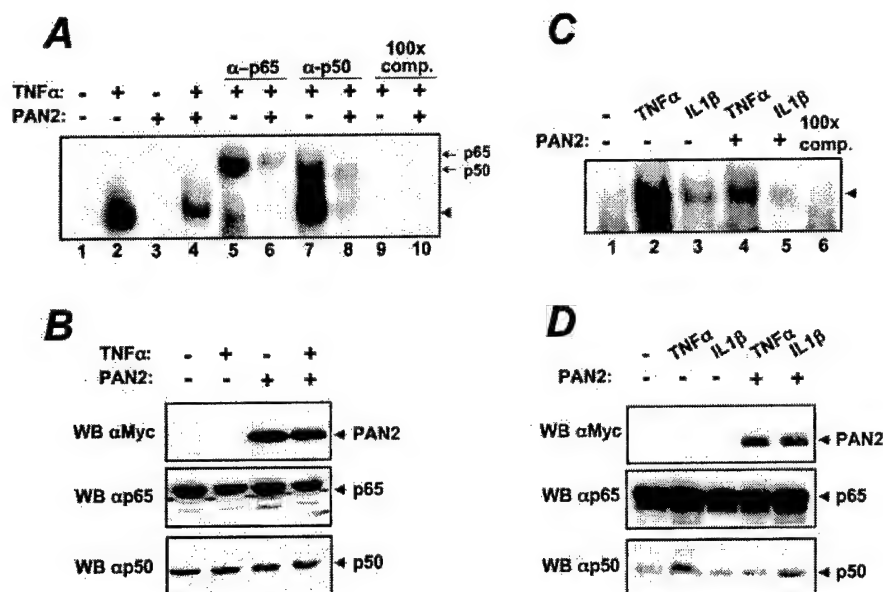


FIG. 4. PAN2 inhibits the DNA binding activity of NF- κ B. **A**, 293-Neo (lanes 1, 2, 5, 7, and 9) or 293-PAN2 (lanes 3, 4, 6, 8, and 10) cells were cultured with (lanes 2, 4, and 5–10) or without (lanes 1 and 3) TNF α (20 ng/ml) for 30 min before producing nuclear extracts. EMSA was performed using 2 μ g of nuclear extracts and a 32 P-labeled double-stranded oligonucleotide probe containing a NF- κ B DNA binding site. Nuclear extracts from stimulated cells were also incubated with specific antibodies recognizing the NF- κ B subunit p65 (lanes 5 and 6) or p50 (lanes 7 and 8). The upper arrows indicate the antibody-shifted complexes. The specificity of the band corresponding to NF- κ B (arrowhead) was determined using a 100-fold molar excess of unlabeled DNA probe as specific competitor (100x comp., lanes 9 and 10). **B**, total cellular extracts from **A** (lanes 1–4) were subjected to immunoblot analysis to confirm equal expression of PAN2, p65, and p50. **C**, as in **A**, EMSA was performed on Myc or Myc-PAN2 stably transfected 293 cells not treated (lane 1) or treated with TNF α (20 ng/ml) for 30 min (lanes 2 and 4) or IL-1 β (100 ng/ml) for 2 h (lanes 3 and 5). The specificity of the band corresponding to NF- κ B (arrowhead) was determined using a 100-fold molar excess of unlabeled DNA probe as specific competitor (100x comp., lane 6). **D**, total cellular extracts from **C** were analyzed by Western blotting as described in **B**. WB, Western blot.

production of the TRAF2, TRAF6, MyD88, RIP, or IRAK proteins in transfected cells (not shown), excluding reduced expression as a trivial explanation for the observations.

NF- κ B induction results from activation of the IKK complex, which phosphorylates the NF- κ B inhibitor I κ B and targets it for ubiquitination and proteasome-mediated degradation, thereby releasing NF- κ B (for review, see Ref. 24). The IKK complex contains two kinases, IKK α and IKK β . These kinases are known to become activated by Nik, a TRAF-binding protein kinase implicated in NF- κ B induction by both TNF and IL-1/Toll family receptors (20). We therefore asked whether PAN2 overexpression alters the ability of Nik, IKK α , and IKK β to induce NF- κ B activity in cells. Using transient transfection and reporter gene assays, similar to the experiment described above, we showed that PAN2 caused dose-dependent suppression of NF- κ B induction by these kinases (Fig. 3C). These observations imply that PAN2 interferes with a distal event at a point of convergence of cytokine signal transduction pathways involved in NF- κ B induction.

To determine whether the observed reduction in NF- κ B activity correlated with reduced NF- κ B DNA binding activity in PAN2-overexpressing cells, an EMSA was performed using a 32 P-labeled double strand DNA oligonucleotide encompassing NF- κ B binding sites. Nuclear extracts were prepared from cells that had been stably transfected with either control or PAN2-encoding plasmids and then stimulated with TNF α or IL-1 β . Incubation of the nuclear extracts with 32 P-labeled NF- κ B probe then permitted measurements of the relative levels of the NF- κ B DNA binding activity. As shown in Fig. 4A, TNF α stimulated increases in NF- κ B DNA binding activity in control transfected cells. In contrast, NF- κ B DNA binding activity was markedly reduced in cells stably overexpressing PAN2. Incubating the protein-DNA complexes with antibodies recognizing various members of the Rel/NF- κ B family of transcription fac-

tors provided evidence that p50 and p65 subunits of NF- κ B are included in the DNA-protein complexes, producing a supershift effect in EMSAs. Similar results were obtained by stimulating the stably transfected cells with IL-1 β (Fig. 4C). Although IL-1 β is less potent than TNF α at inducing NF- κ B in these cells, the extent of inhibition by PAN2 was comparable for these two cytokines. Immunoblot analysis of cell lysates revealed that 293-Neo and 293-PAN2 cell lines express equivalent levels of NF- κ B p50/p65 proteins, excluding the possibility that a reduced level of these proteins accounts for the results (Fig. 4, B–D). We conclude from these experiments that PAN2 inhibits the induction of NF- κ B DNA binding activity by TNF α and IL-1 β .

The PAAD Domain of PAN2 Is Sufficient for Suppression of NF- κ B Activity—To explore the region within PAN2 responsible for inhibition of NF- κ B activation, we constructed plasmids encoding Myc-tagged deletion mutants of PAN2 containing the PAAD domain alone (aa 1–94), a mutant lacking the PAAD domain (Δ PAAD, aa 147–994), a mutant lacking the leucine-rich repeats (Δ LRR, aa 1–620), and a mutant containing only the LRRs (aa 605–994) (Fig. 5A). These plasmids were then transiently expressed in HEK293 cells in equal amounts using a plasmid dose at which PAN2 suppresses TNF α induction of NF- κ B by ~50% so that either loss or gain of function could be detected. As shown in Fig. 5B, TNF α -mediated NF- κ B activation was inhibited to similar extents by full-length PAN2, the PAAD domain only, and PAN2 lacking the LRRs. In contrast, the PAN2 mutant lacking the PAAD domain displayed reduced activity, and a mutant encompassing only the LRR was completely inactive at suppressing TNF α -mediated induction of NF- κ B activity. The failure of the LRR region to inhibit TNF α -mediated NF- κ B induction was not due to a failure to produce comparable amounts of this protein compared with the other PAN2 mutants as demonstrated by immunoblot analysis (Fig. 5C).

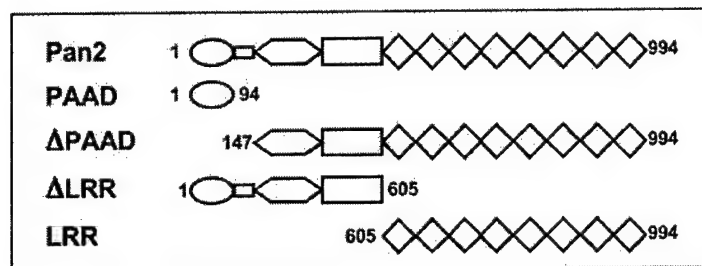
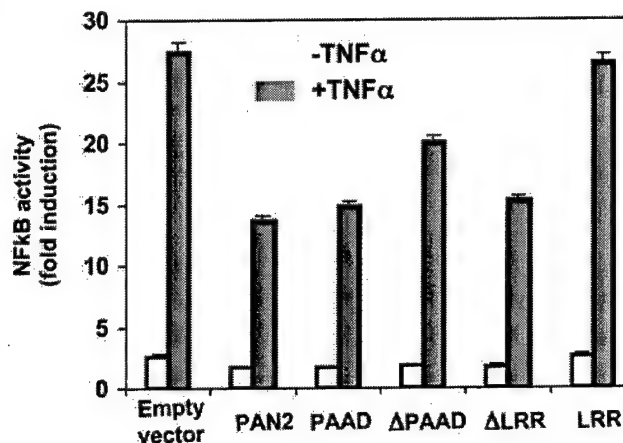
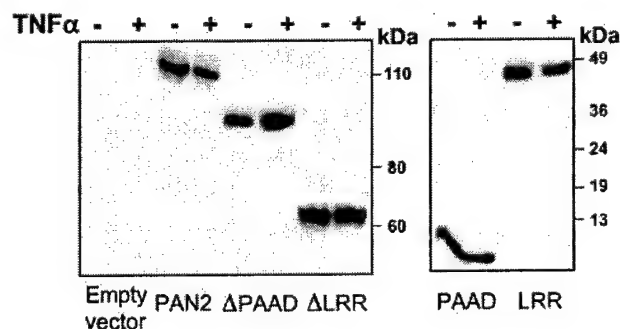
A

FIG. 5. Structure-function analysis of PAN2. **A**, a schematic representation of Myc-tagged PAN2 deletion mutants is depicted. **B**, HEK293 cells were transfected with 50 ng of pNF- κ B-luc, 10 ng of pTK-RL reporter vectors, and either 400 ng of pcDNA3Myc empty vector or the same amount of the indicated Myc-tagged PAN2 deletion mutants. At 36 h after transfection, cells were either left untreated (white bars) or stimulated for 6 h with TNF α (gray bars). Cells were then harvested, and luciferase activities were determined as described under "Materials and Methods." Data indicate the -fold induction of luciferase activity (mean \pm S.D., $n = 3$). **C**, immunoblot analysis of PAN2 mutants was performed. Lysates from transfected HEK293 cells were normalized for total protein content (30 μ g) and subjected to SDS-PAGE/immunoblot analysis using anti-Myc antibody with enhanced chemiluminescence-based detection. Molecular mass markers are indicated at right.

B**C**

PAN2 Associates with and Inhibits Activation of IKKs—Since the functional analysis of PAN2 suggested that it can suppress NF- κ B activity induced by overexpression of IKK α or IKK β , we explored whether PAN2 might associate with components of the IKK complex. Using lysates from HEK293 cells in which PAN2 was co-expressed with epitope-tagged IKK α , IKK β , or IKK γ , co-immunoprecipitation assays were performed, revealing association of IKK α with PAN2 (Fig. 6A). Under these conditions, association of PAN2 with IKK β or IKK γ was not detected. Immunoblot analysis of the lysates confirmed production of IKK α , IKK β , and IKK γ at comparable levels, excluding differences in the levels of expression of these proteins as an explanation for the selective association with IKK α . We were also able to detect the interaction of PAN2 with the endogenous IKK α (Fig. 6B). In contrast to IKK α , PAN2 did not co-immunoprecipitate with other proteins such as p105 or Nik (not shown), further demonstrating specificity.

Next we measured the effect of PAN2 on the activity of IKK α

using *in vitro* kinase assays. For these experiments, HEK293 cells were transiently transfected with plasmids encoding epitope-tagged IKK α or IKK β together with either a control plasmid or various amounts of a PAN2-encoding plasmid. After 36 h, cells were left untreated or stimulated with TNF α for 15 min, then cell lysates were prepared, and either IKK α or IKK β was immunoprecipitated. The resulting immunoprecipitates were then used for *in vitro* kinase assays where they were incubated with the exogenous substrate (GST-I κ B α -(1-54)) in the presence of [γ - 32 P]ATP. The kinase reaction products were then analyzed by SDS-PAGE, examining phosphorylation of GST-I κ B α substrate as well as phosphorylation of the kinases. Furthermore, the immunoprecipitates were subjected to SDS-PAGE/immunoblot analysis to verify loading of equivalent amounts of proteins.

As shown in Fig. 7A, TNF α induced increases in both the phosphorylation and kinase activity of IKK α in control-transfected cells. In contrast, TNF α -inducible IKK α activity and

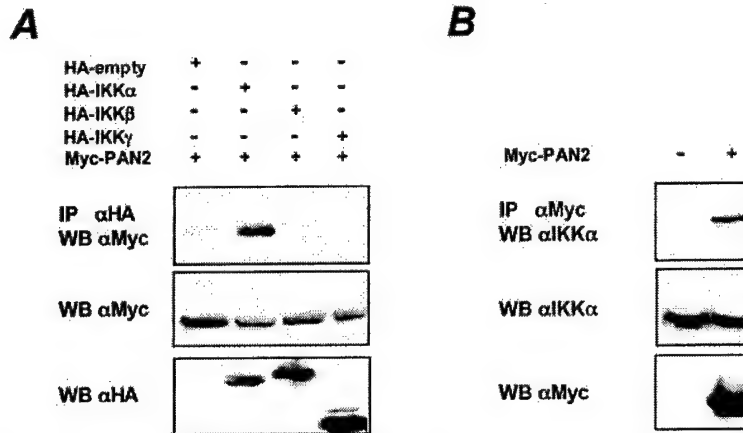


FIG. 6. PAN2 interacts with IKK α . A, 293T cells were transfected with 4 μ g of pcDNA3Myc-PAN2 together with 4 μ g of HA empty vector (lane 1) or 4 μ g of plasmids encoding HA-IKK α , HA-IKK β , or HA-IKK γ (lanes 2–4). After 36 h, cell lysates were prepared and subjected to immunoprecipitation (IP) with anti-HA antibody. The resulting immune complexes were then analyzed by SDS-PAGE/immunoblotting using anti-Myc antibody (top). Alternatively lysates were run directly on gels (25 μ g of protein) and analyzed by immunoblotting (WB) using anti-Myc antibody (middle) or anti-HA (bottom) to confirm expression of proteins. B, 293-Neo or 293-PAN2 stable cell lysates were immunoprecipitated (IP) with anti-Myc antibody. Immune complexes were then analyzed by immunoblotting using anti-IKK α antibody (Santa Cruz Biotechnology) to detect the association of PAN2 with the endogenous IKK α (top). Alternatively lysates were run directly on gels (25 μ g of protein) and analyzed by immunoblotting (WB) using anti-IKK α (middle) or anti-Myc (bottom) to confirm expression of proteins. WB, Western blot.

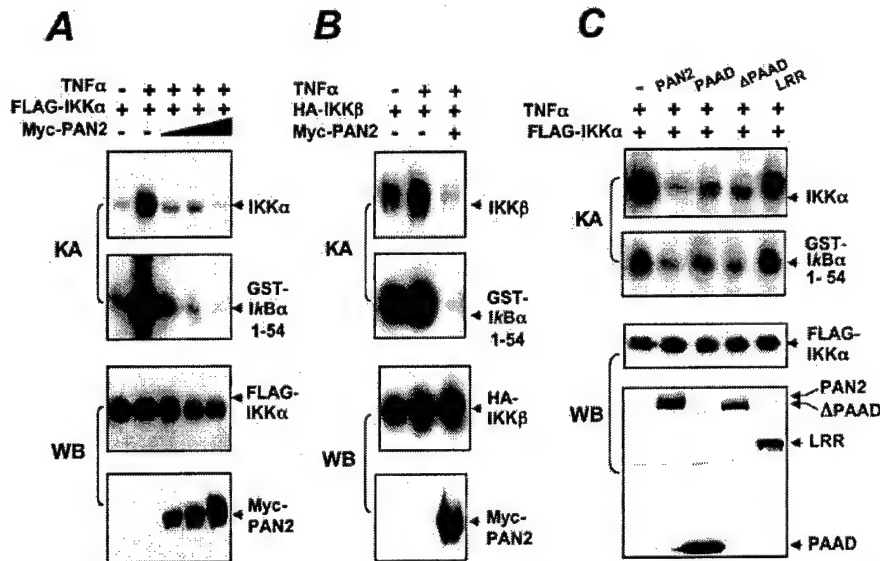


FIG. 7. PAN2 inhibits IKKs activity. A, HEK293 cells were co-transfected with 0.5 μ g of FLAG-IKK α and either pcDNA3Myc empty vector (lanes 1 and 2) or increasing amounts (0.5, 1, and 2.5 μ g) of Myc-PAN2 (lanes 3–5). After 36 h, cells were cultured with (+) or without (–) 20 ng/ml TNF α for 15 min, and cell lysates were prepared for immunoprecipitation with anti-FLAG antibody. Immune complexes were used for *in vitro* kinase assays (KA) measuring IKK α phosphorylation (top) and phosphorylation of exogenous substrate GST-I κ B α (1–54) (second from top). Immunoblot analysis of the immune complexes with anti-FLAG antibody (third from top) and of lysates with anti-Myc antibody (bottom) was performed to contrast the amounts of FLAG-IKK α immunoprecipitated and the relative amounts of Myc-PAN2 produced, respectively. B, similar experiments were performed for IKK β using 0.5 μ g of HA-IKK β - and 2.5 μ g of Myc-PAN2-encoding plasmids. C, HEK293 cells were co-transfected with 0.5 μ g of FLAG-IKK α and 0.5 μ g of either pcDNA3Myc empty vector (lane 1) or the same amount of the indicated Myc-tagged PAN2 deletion mutants (lanes 2–5). Immunoprecipitations and kinase assays as well as immunoblot assays were performed as described above.

phosphorylation were suppressed in a concentration-dependent manner by PAN2. Similar results were obtained for IKK β where PAN2 overexpression potentially suppressed IKK β activity below base-line levels in TNF α -stimulated HEK293 cells (Fig. 7B).

To determine whether the PAAD domain of PAN2 is necessary for inhibition of IKK α activity, we compared the effects of full-length PAN2 with deletion mutants of PAN2 comprising the PAAD alone or lacking the PADD domain (Δ PAAD). As an additional control, a fragment of PAN2 representing only the LRRs was also tested. Plasmids encoding full-length and deletion mutants of PAN2 were transfected into 293 cells together with FLAG-tagged IKK α (in a 1:1 ratio), and kinase assays were performed as described above. As shown in Fig. 7C, both

PAAD-only and Δ PAAD mutants inhibited IKK α activity (measured by *in vitro* phosphorylation of IKK α and GST-I κ B α (1–54)), although somewhat less potently than full-length PAN2. In contrast, a mutant encompassing only the LRR was almost inactive at suppressing IKK α activity, although all mutants were expressed to a comparable levels (Fig. 7C, bottom panel). We conclude that the PAAD domain is sufficient to suppress NF- κ B, but other regions of the PAN2 protein can also interfere with cytokine-mediated induction of NF- κ B.

DISCUSSION

This report provides evidence that the PAAD-containing protein PAN2 regulates NF- κ B activity by affecting the I κ B ki-

nases. Gene transfer-mediated increases in the levels of PAN2 suppressed NF- κ B transcriptional activity in response to TNF α and IL-1 β , implying that PAN2 operates at a point of convergence of these cytokine signal transduction pathways. Consistent with this hypothesis, PAN2 also suppressed induction of NF- κ B activity by several signal transduction mediators within the TNF and IL-1 receptor pathways, including TRAF2, TRAF6, RIP, IRAK2, and NIK. Furthermore, PAN2 associates with IKK α and suppresses cytokine-mediated activation of this kinase, which plays a critical role in controlling degradation of I κ B, thus releasing NF- κ B. Although PAN2 did not associate with IKK β in co-immunoprecipitation assays, it nevertheless suppressed its activation. This observation is consistent with reports that have suggested that IKK α operates upstream of IKK β in some cytokine signaling pathways (25). At this point, we do not know whether the association of PAN2 with IKK α is direct *versus* indirect, requiring additional proteins analogous to the structurally similar CARD family proteins Nod1 and Nod2, which interact with the IKK complex indirectly via the adapter protein Cardiak (RIP2, RICK) (26). Indirect association might explain why only IKK α was co-immunoprecipitated with PAN2 instead of the entire IKK complex of IKK α , IKK β , and IKK γ . In this regard, it is also possible that interaction of PAN2 with IKK α dissociates the IKK complex, explaining why PAN2 suppresses activation of both IKK α and IKK β .

The architecture of PAN2 is similar to multiple proteins in animals and plants that contain various N-terminal effector domains followed by a NACHT domain and LRRs (27). The N-terminal effector domains range from CARDs in mammalian Nod1 (CARD4), Nod2 (inflammatory bowel disease protein 1), and CLAN (Ipaf, CARD12) to leucine zippers and Toll/IL-1 receptor domains in plants. Many of these proteins are presumably involved in innate immunity where their LRRs bind ligands produced by bacterial pathogens. For Nod1 and Nod2, for instance, it has been suggested that their LRRs bind lipopolysaccharide, triggering activation of the Nod1 and Nod2 proteins and inducing NF- κ B (28, 29). One model for how these proteins become activated envisions the unliganded LRRs functioning as negative regulatory domains that suppress oligomerization of the NACHT domains until appropriate stimulatory ligands bind, relieving this autorepression. If PAN family proteins such as Cryopyrin and PAN2 operate in the same way, then we might expect their LRRs to recognize pathogen products, changing the activity state of these proteins. For this reason, we cannot exclude the possibility that PAN2 functions as a stimulator rather than inhibitor of NF- κ B under some circumstances. However, as shown here, a truncation mutant of PAN2 lacking the LRRs suppressed (rather than enhancing) TNF α -mediated induction of NF- κ B activity. Nevertheless, a recent report suggested that Cryopyrin can activate NF- κ B when co-expressed with the PAAD/CARD protein ASC (30). In contrast, we have been unable to detect interactions of PAN2 with ASC.³

If PANs operate as suppressors of NF- κ B *in vivo*, then one might speculate that the hereditary mutations associated with the PAN family protein Cryopyrin and the PAAD-containing protein Pyrin alter the functions of these proteins so that they are no longer capable of properly suppressing NF- κ B, thereby explaining the hyperinflammatory syndromes associated with mutations in the genes encoding these proteins (21). We there-

fore speculate that at least some members of the PAAD family function in a negative feedback mechanism that ensures that NF- κ B activity is produced in short bursts that limit inflammatory responses. It seems likely that PAADs may function as either inducers or suppressors of NF- κ B depending on the balance of homotypic interactions between the PAAD domains of this large family of proteins, which presumably set thresholds within cells for NF- κ B induction and inflammatory responses. In this regard, we analyzed the levels of endogenous PAN2 in HeLa and 293 cells after stimulation with TNF α , IL-1 β , lipopolysaccharide, or phorbol 12-myristate 13-acetate, but no change in expression levels was observed (data not shown), suggesting that PAN2 protein is not induced by stimuli known to trigger NF- κ B activation in these cell lines. Thus PAN2 activation may occur through a mechanism that involves modification of the protein at a post-translational level, its translocation in a particular cellular compartment, or its interactions with other proteins. Further work is needed to explore how PAN2 is regulated in response to inflammatory stimuli and to determine the specific biological contexts in which PAN2 operates.

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³ L. Fiorentino, C. Stehlik, and J. C. Reed, unpublished data.

Brief Definitive Report**Nuclear Factor (NF)- κ B-regulated X-chromosome-linked *iap* Gene Expression Protects Endothelial Cells from Tumor Necrosis Factor α -induced Apoptosis**

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Summary

By differential screening of tumor necrosis factor α (TNF- α) and lipopolysaccharide (LPS)-activated endothelial cells (ECs), we have identified a cDNA clone that turned out to be a member of the inhibitor of apoptosis (*iap*) gene family. *iap* genes function to protect cells from undergoing apoptotic death in response to a variety of stimuli. These *iap* genes, *hiap1*, *hiap2*, and *xiap* were found to be strongly upregulated upon treatment of ECs with the inflammatory cytokines TNF- α , interleukin 1 β , and LPS, reagents that lead to activation of the nuclear transcription factor κ B (NF- κ B). Indeed, overexpression of I κ B α , an inhibitor of NF- κ B, suppresses the induced expression of *iap* genes and sensitizes ECs to TNF- α -induced apoptosis. Ectopic expression of one member of the human *iap* genes, human X-chromosome-linked *iap* (*xiap*), using recombinant adenovirus overrules the I κ B α effect and protects ECs from TNF- α -induced apoptosis. We conclude that *xiap* represents one of the NF- κ B-regulated genes that counteracts the apoptotic signals caused by TNF- α and thereby prevents ECs from undergoing apoptosis during inflammation.

Key words: activation • inhibitor of apoptosis gene family • endothelial cells • adenovirus • nuclear factor κ B

Endothelial cells (ECs) are located at the strategic interface between blood stream and tissue and regulate local exchange of cells and nutrients. They are critically involved in local and systemic inflammatory responses at the sites of transmigration of immune cells such as neutrophils, monocytes, and lymphocytes. The concentration of inflammatory cytokines at the site of transmigration is expected to be high, and in fact inflammatory cytokine-mediated activation of ECs is responsible for the attraction, adhesion, and extravasation of white blood cells to the inflamed tissue.

Stimulation of cells with TNF- α , a potent inflammatory cytokine, generates two types of signals: one that initiates programmed cell death (1), and one that leads to activation of the transcription factor NF- κ B (2), and subsequently to the inflammatory response. The overall result in a specific cell type is dependent on the balance of the two signals. Direct inhibition of NF- κ B or of the upstream parts of its signaling pathway during TNF- α activation results in apoptosis in a variety of cell types originally resistant to TNF- α -induced apoptosis (3, 4). Furthermore, fibroblasts and macrophages from NF- κ B subunit p65-deficient mice are more sensitive to TNF- α -induced apoptosis (5). Therefore, it has been proposed that activation of NF- κ B induces

the expression of genes that counteract apoptotic signals and prevent cell death.

Members of the inhibitor of apoptosis (*iap*) gene family have been demonstrated to suppress apoptosis induced by a variety of stimuli in different cell types (6-13, and for review see reference 14). The *iap* genes have also been shown to play a role in TNF- α -induced programmed cell death. Different *iap* gene family members appear to interfere with the cell death-triggering cascade at different levels. *hiap1* and *hiap2* can bind to the TNFR-associated factor 2 (TRAF2), a molecule that is associated with the cytoplasmic part of the TNFR complex and is essential for the activation of NF- κ B (9, 15). Both have also been shown to be direct inhibitors of cell death proteases caspase 3 and caspase 7 (16). Another *iap* gene family member, the X-chromosome-linked *iap* (*xiap*), protects embryonic kidney 293T cells from bax-triggered apoptosis by inhibiting the same proteases, but in contrast it has not been found to be associated with members of the TRAF family (16, 17).

The studies presented here demonstrate that three human *iap* gene family members (*xiap*, *hiap1*, and *hiap2*) are strongly upregulated in TNF- α -stimulated primary ECs, which are resistant to TNF- α -induced apoptosis. How-

ever, adenovirus-mediated overexpression of I κ B α (18, 19), an inhibitor of NF- κ B, renders primary ECs sensitive to TNF- α -induced apoptosis and at the same time inhibits *iap* gene upregulation. Thus, *iap* gene expression appears to be dependent on NF- κ B activation. Importantly, we show that ectopic expression of *xiap* is sufficient to overcome the I κ B α effect in I κ B α -overexpressing ECs and protects these cells from TNF- α -induced apoptosis.

Materials and Methods

Cell Culture

Cell culture flasks were coated with 1% gelatine for 30 min at 37°C. Human umbilical vein endothelial cells (HUVECs) and human skin microvascular endothelial cells (HSMECs) were grown in medium M199 supplemented with 20% bovine calf serum (HyClone, Logan, UT), endothelial cell growth factor supplement (Technoclon, Vienna, Austria), penicillin, streptomycin, fungizone, and heparin (3 U/ml). Confluent cells were split in a 1:3 ratio and used up to the sixth passage.

U937 cells were cultivated in RPMI-1640 medium supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. Cells were split 1:10 when grown to a density of 10^6 cells/ml.

Northern Analysis

Total RNA was isolated using Trizol reagent (GIBCO BRL, Gaithersburg, MD). 10 μ g total RNA was separated on a 1.3% formaldehyde agarose gel. Samples were run in 0.02 M MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0, 5 mM sodium acetate, 1 mM EDTA. The gel was blotted overnight using $10\times$ SSC onto a GeneScreen Plus nylon membrane (Dupont-NEN, Boston, MA), dried, and fixed by UV-light (UV-cross-linker 120.000 μ J; Stratagene Inc., La Jolla, CA). Membranes were hybridized with α -[32 P]dATP-labeled (Terminal Transferase, Boehringer Mannheim, Mannheim, Germany) oligonucleotides specific to *hiap1* (5'-agaatgttcagttggcattcaatcaacccaagatgtaagtgtgactcatgaagcttct-3'), *hiap2* (5'-aagatttcaccacaaaagaagaatcaatgatagactcttatgtagaattactacacttc-3'), *xiap* (5'-gaagggtggtgggtgggaacaacacagctccctaggaagagcacaggatgtcacggggg-3'), and *naip* (5'-actgcatctaggccagaagagcagacagctctggcagcaattgtgatcaaatgggaga-3') using Quickhyb-solution (Stratagene, Inc.) at 65°C. Membranes were washed twice for 15 min at room temperature in 1% SDS/3 \times SSC/20 mM sodium phosphate buffer, pH 7.2, and twice for 30 min at 65°C in 1% SDS/1 \times SSC. Signals were analyzed on a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

Adenovirus Construction and Infection

Adenovirus I κ B α has been described previously (26), and construction of *xiap* adenovirus was done by firstly introducing a fragment encoding the myc peptide sequence MEQKLISEEDL into the adenovirus transfer vector pACCMVpLpASR+ (20). Subsequently, a 1,600-bp BamHI/XbaI cDNA fragment containing the entire coding region of human *xiap* was ligated and the construct was cotransfected together with pJM17, a plasmid containing the adenoviral genome with a deletion in the E1 region into 293 cells (21). Plaques appearing after 10 d of culture were subcloned on 293 cells and were tested for *xiap* expression on immunoblots using anti-myc mAb 9E10 (22). Purification of a large batch of the recombinant adenovirus was done by two consecutive cesium chloride centrifugations as previously described (23).

Postconfluent HSMECs and HUVECs were washed once with complete PBS and incubated at a multiplicity of infection of

100 with the respective adenovirus constructs in PBS. After 30 min at 37°C, the adenovirus was washed off and fresh medium was added. Cells were maintained for an additional 2 d before being assayed.

Analysis of DNA Fragmentation

Electrophoresis of Genomic DNA. Cells were incubated for 3 h at 55°C (100 mM NaCl, 10 mM Tris HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.47 mg/ml Proteinase K), and then incubated with 100 μ g/ml RNaseA for 1 h at 37°C. After phenol-chloroform extraction and isopropanol precipitation, the DNA was dissolved in 50 μ l Tris/EDTA and resolved on 1.3% agarose gel.

Quantification of Fragmented DNA. For quantification of apoptosis fragmented DNA was determined by sandwich ELISA with antihistone coated microtiter plates and peroxidase-conjugated anti-DNA antibodies using the Cell Death Detection ELISA system from Boehringer Mannheim, according to the manufacturer's protocol.

Flow Cytometry

48 h after infection cells were treated with TNF- α (500 U/ml) for 6 h or left untreated. Cells were harvested, fixed in 70% ethanol, and the proportion of cells undergoing apoptosis was determined by flow cytometric analysis (FACSORT[®], Becton Dickinson, San Jose, CA) after staining with propidium iodide. Cells with a DNA content <2 N appear in the sub-G1 region (M1).

Results and Discussion

Using a modified differential screening technique to identify and clone genes regulated by inflammatory mediators in porcine aortic ECs (PAECs) (23a) we have obtained a porcine homologue (*piap*) of the human *iap* gene family. Initially identified as a TNF- α -inducible gene, *piap* was found also to respond to the inflammatory stimuli LPS and to a lesser degree to IL-1 β . Subsequently, we have tested whether members of the human *iap* gene family (*xiap* [hILP, MIHA], *hiap1* [ciap2, MIHC], and *hiap2* [ciap1, MIHB]; references 6–12) show similar responses to inflammatory cytokines. Using oligonucleotides specific for the different *iap* genes, we performed Northern blot analysis of HSMECs (Fig. 1) and HUVECs (data not shown). We demonstrate that, apart from the neuronal inhibitor of apoptosis (*naip*) that is not expressed in ECs, the *xiap*, *hiap1*, and *hiap2* genes were strongly upregulated in response to TNF- α in HSMECs and HUVECs.

Treatment of HSMECs or HUVECs with TNF- α for up to 24 h did not lead to apoptosis, whereas the well-established TNF- α -sensitive monocytic cell line U937 became apoptotic under these experimental conditions. *iap* gene expression has been shown to inhibit apoptosis induced by a variety of apoptotic stimuli (12). Thus, we speculated that induced *iap* gene expression may prevent ECs from undergoing programmed cell death in response to TNF- α .

TNF- α is a proinflammatory cytokine whose pleiotropic biological effects are signaled through two distinct cell surface receptors, TNFR 1 and TNFR 2 (2). It is known to be a potent activator of NF- κ B that has been shown to be the central mediator of gene regulation in the inflammatory

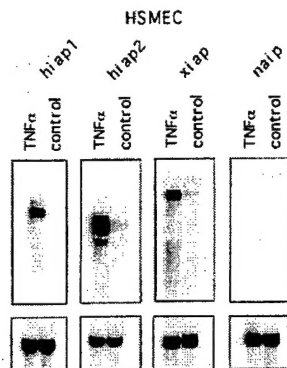


Figure 1. Northern blot analysis of *iap* gene expression in HSMECs. 10 μ g of total RNA from either nontreated or TNF- α -treated (500 U/ml) HSMECs was loaded in each lane and hybridized to oligonucleotides specific to *hiap1*, *hiap2*, *xiap*, and *naip*. The predicted transcript size corresponds to the published one (7) for *hiap1* (6.5 kb), *hiap2* (4.5 kb), and *xiap* (9 kb). To confirm the equal loading of RNA, membranes were stripped and reprobed with GAPDH.

response of activated ECs leading to leukocyte adhesion and thrombosis (24, 25). Therefore, we tested whether NF- κ B was involved in upregulation of *iap* genes in response to inflammatory stimuli. Having shown previously that expression of I κ B α from a recombinant adenovirus vector abolishes NF- κ B-dependent upregulation of inflammatory genes such as IL-1 β , IL-6, IL-8, and vascular cell adhesion molecule 1 in LPS-stimulated ECs (26), we used this adenovirus-I κ B α construct to investigate whether NF- κ B inhibition also impairs *iap* gene expression. HUVECs and HSMECs were infected with either a control adenovirus or the recombinant adenovirus I κ B α (27). After 2 d, cells were stimulated with TNF- α for 4 h and probed for *xiap*, *hiap1*, and *hiap2* expression. As shown in Fig. 2, the expression of all three *iap* genes tested in adenovirus I κ B α -infected ECs was suppressed, indicating that the upregulation of *iap* genes is controlled by activation of NF- κ B.

We then raised the question whether blocking the activation of NF- κ B would actually sensitize ECs to TNF- α -induced apoptosis. Indeed, ECs infected with the recombinant adenovirus I κ B α construct started to die \sim 6 h after TNF- α stimulation. To demonstrate that the apoptotic program is involved in cell death, genomic DNA was isolated from dying cells. As shown in Fig. 3, genomic

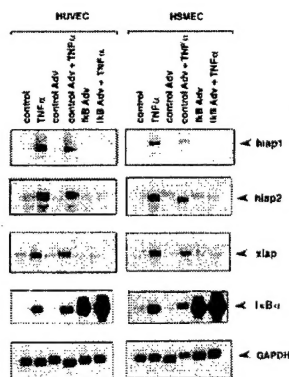


Figure 2. Northern blot analysis of *iap* gene expression in adenovirus I κ B α -infected ECs. HUVECs and HSMECs were not infected, were infected with a control adenovirus, or were infected with the recombinant adenovirus I κ B α construct. Cells were either left untreated or treated with TNF- α (500 U/ml) for 4 h. The membranes were probed with oligonucleotides specific to *hiap1*, *hiap2*, and *xiap*. Expression of I κ B α was controlled by reprobing the membranes with an I κ B α -cDNA. Equal loading was confirmed by hybridization with a GAPDH cDNA probe. Adv, adenovirus.

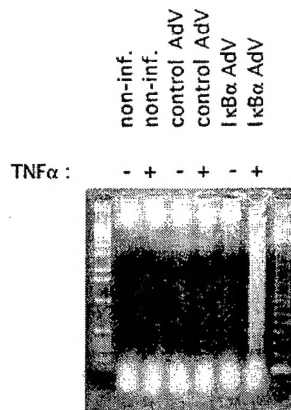


Figure 3. DNA fragmentation in adenovirus I κ B α -infected and TNF- α -stimulated HUVECs. HUVECs were not infected, were infected, with a control adenovirus, or were infected with the recombinant adenovirus-I κ B α construct. Noninfected cells and infected cells were left untreated or treated with TNF- α (500 U/ml) for 6 h. Appearance of fragmented genomic DNA was analysed by 1.3% agarose gel electrophoresis. Left lane: 1-kb ladder molecular weight standard; right lane: 123-bp ladder molecular weight standard. Non-inf., noninfected cells; Adv, adenovirus.

DNA from I κ B α -expressing and TNF- α -treated cells, but not from control virus-infected or nontreated cells, showed the DNA fragmentation pattern characteristic for apoptosis. Thus, inhibition of NF- κ B activation renders ECs TNF- α sensitive, indicating that induction of apoptosis in ECs can occur independent of NF- κ B.

These data suggested that TNF- α -induced expression of *iap* genes could be required to protect ECs from undergoing apoptosis. To directly demonstrate the ability of *iap* genes to prevent ECs from TNF- α -induced apoptosis, we coinfect HUVECs with recombinant adenovirus constructs expressing myc-tagged *xiap* and I κ B α , respectively. Infection with recombinant adenovirus I κ B α alone and stimulation with TNF- α -induced apoptosis in HUVECs (Fig. 4 B, c and d). Coexpression of *xiap* and I κ B α (Fig. 4 B, f) reduced the percentage of apoptotic cells to background levels obtained in TNF- α -treated or nontreated HUVECs (Fig. 4 B, a and b). A recombinant adenovirus expressing green fluorescent protein (27) was used as a control to show that adenovirus infection itself had no influence on apoptosis induced by TNF- α in I κ B α -overexpressing cells (Fig. 4 B, g and h). Expression of myc-tagged *xiap* in infected HUVECs was demonstrated by Western blots stained with anti-myc mAb (Fig. 4 A).

Since the monocytic cell line U937 is sensitive to TNF- α -induced apoptosis when compared to primary ECs, we analyzed whether this cell line also differs with respect to TNF- α -inducible upregulation of *iap* genes. U937 and HUVECs were treated with TNF- α for 4, 6, and 9 h. At the same time points we monitored and quantified apoptosis by analysis of fragmented genomic DNA using an ELISA assay for histone-associated DNA fragments. Fig. 5 C shows that *xiap* gene was barely expressed in nontreated U937 cells and expression could not be induced by TNF- α . Consistently, U937 cells became significantly apoptotic after 4 h (Fig. 5 D). In contrast, as shown in Fig. 5, A and B, *xiap* was upregulated in HUVECs and no increase in fragmented DNA could be assayed in response to TNF- α . Identical results were obtained for *hiap1* and *hiap2* in U937 cells and HSMECs (data not shown).

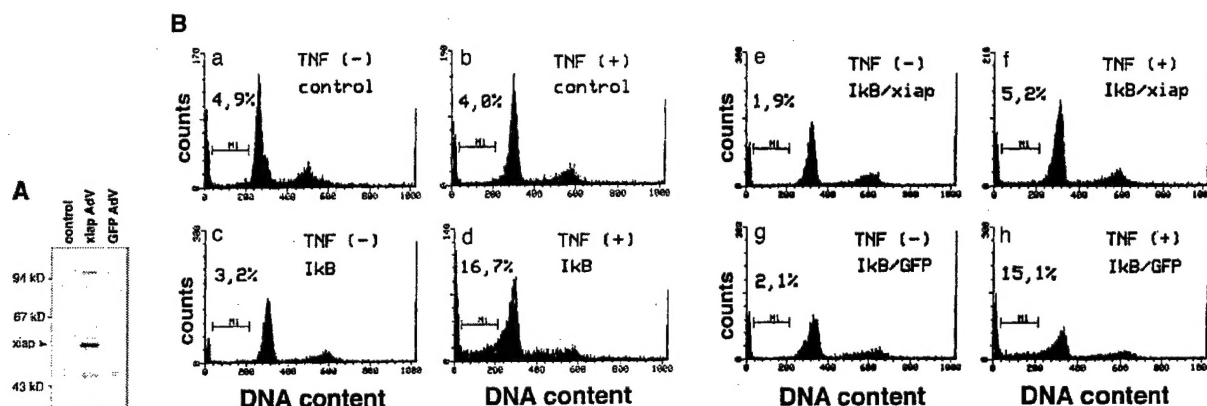


Figure 4. Inhibition of apoptosis by ectopic *xiap* expression. (A) Lysates of noninfected or infected HUVECs were separated by SDS-PAGE, blotted onto nylon membranes, and stained for myc-tagged XIAP protein. *AdV*, adenovirus; *GFP*, green fluorescent protein. (B) HUVECs were infected with IκBα alone (e and f), together with *xiap* (e and f), or together with GFP (g and h) recombinant adenovirus. 48 h after infection cells were treated with TNF-α (500 U/ml) for 6 h or left untreated and analyzed by FACS[®] after propidium iodide staining. Cells with a DNA content <2 N appear in the sub-G1 region (M1). The percentage of cells found in the M1 region is indicated. The data show one out of three representative experiments.

Our findings provide several lines of evidence that the *iap* gene products are regulated by NF-κB and that *xiap* appears to be sufficient to protect primary ECs from undergoing apoptosis in response to TNF-α: (a) *iap* genes are expressed in response to TNF-α, IL-1β, and LPS, respectively; (b) inhibition of NF-κB activation suppresses inducible *iap* gene expression; (c) inhibition of NF-κB activation by

overexpressing its inhibitor IκBα renders ECs sensitive to TNF-α-induced apoptosis; and (d) ectopic expression of *xiap* in IκBα-overexpressing ECs overrides the IκBα/TNF-α effect.

These data show that ECs and presumably other cells have developed cellular mechanisms that protect them from apoptosis and keep them able to function properly in an inflammatory situation. Fast activation of NF-κB in response to proinflammatory signals, like TNF-α, would be an appropriate mechanism to ensure the prompt expression of antiapoptotic gene(s). This hypothesis is supported by the demonstration that NF-κB p65 is necessary to protect fibroblasts from TNF-α-induced apoptosis (5).

Whether under physiological circumstances the expression of *xiap* is sufficient or whether simultaneous expression of all three *iap* genes (or other genes such as A20 [28], manganese superoxide dismutase [29], plasminogen activator-inhibitor type 2 [30], A1 [31], or other as yet undefined genes) is required to protect ECs from TNF-α-induced apoptosis remains open. Chu et al. (32) have shown recently that *hiap1* expression is dependent on activation of NF-κB in Jurkat cells and *hiap1* protein is able to protect these cells from apoptosis. However, in contrast to primary ECs, *hiap2* showed a steady state level of expression in Jurkat cells and was not controlled by NF-κB. The data indicate that expression of the *iap* gene family members and their involvement in protection from apoptosis varies in certain cell types and follows a rather complex scheme. *iap* gene expression appears to be specific for the cell type and the given stimulus. This view is supported by our finding that *iap* gene expression seems to be not involved in the TNF-α response of the monocytic cell line U937. These cells become partially apoptotic upon TNF-α treatment but do not express *iap* genes, suggesting that other protective mechanisms are operative. Recent reports demonstrated that *hiap1/2* can interfere at different levels with the apoptotic program. *hiap1* and *hiap2* associate via TRAF 2

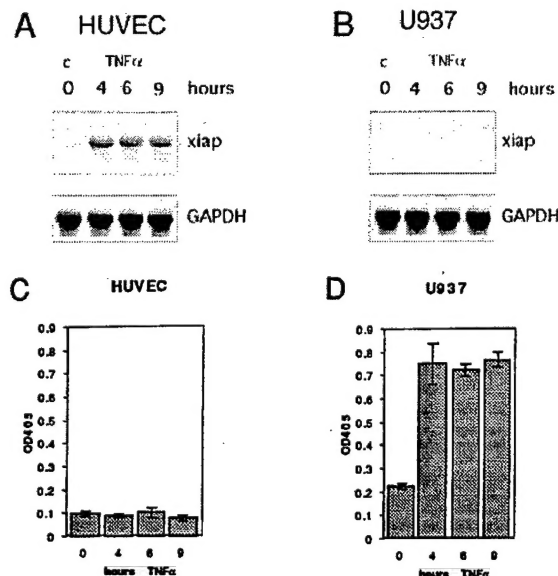


Figure 5. Lack of TNF-α-inducible *xiap* gene expression correlates with apoptosis in U937 cells. Northern blot analysis of *xiap* gene expression in HUVECs (A) and U937 cells (C). HUVECs and U937 cells were treated for 4, 6, and 9 h with TNF-α. To confirm equal loading of RNA, membranes were stripped and reprobed with GAPDH. TNF-α-induced genomic DNA fragments from HUVECs (B) and U937 cells (D) were determined by colorimetric enzyme immunoassay. Columns represent the mean of three independent experiments. SD is indicated by error bars. TNF-α (500 U/ml); c, nontreated cells.

with the TNFR 2, leading to NF- κ B activation (9), and *hiap2* is also part of the TNFR 1 signaling complex (15). On the other hand, *hiap1* and *hiap2* as well as *xiap* directly inhibit caspase 3 and caspase 7 activity, two members of the caspase family of cell death proteases, in embryonic kidney 293T cells (16, 17). However, inhibition by *xiap* is two to three orders of magnitude more potent, suggesting *xiap* as the physiological inhibitor of caspase 3 and 7 (16). These data and our finding that *xiap* expression is sufficient to prevent TNF- α -induced apoptosis in ECs support the concept that *xiap* plays a central role in inhibition of programmed cell death. It remains to be established whether *xiap* operates via an identical mechanism in ECs as in 293T cells and which other cell-type specific and stimulus-dependent mechanisms exist.

Unexpectedly, *iap* gene expression is also induced by LPS and IL-1 β . Pretreatment of a human fibrosarcoma line (HT1080V) with the nonapoptotic, NF- κ B-inducing IL-1 protects these cells from apoptosis induced by the later addition of TNF- α even in the presence of a protein synthesis inhibitor (3). In cells expressing a super-repressor form of the NF- κ B inhibitor I κ B α , IL-1 β does not have this protective effect, suggesting that IL-1 β also induces the expression of NF- κ B-regulated antiapoptotic genes. A mechanism to overrule apoptotic signals during inflammation would enable ECs to respond properly by upregulation of inflammatory mediators such as tissue factor and adhesion molecules and at the same time to survive inflammation in order to maintain homeostasis of the inflamed tissue and initiate the healing process.

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